Induced pluripotent stem cell-derived neural progenitor cell transplantation promotes regeneration and functional recovery after post-traumatic stress disorder in rats

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ABSTRACT

Post-traumatic stress disorder (PTSD) is a mental disorder characterized by hippocampal neuron loss and cognitive dysfunction. The aim of the present study was to investigate the potential functional outcomes of transplantation of induced pluripotent stem cell-derived neural progenitor cells (iPSC-NPCs) for treating PTSD. Human induced pluripotent stem cell (iPSCs), differentiated into neural progenitor cells (NPCs) *in vitro*, were transplanted into the brain of rat. Following iPSC-NPCs transplantation, cognitive function was determined. The open field test and fear condition test indicated that long-term iPSC-NPCs transplantation ameliorated cognitive dysfunction and reduced freezing time in PTSD rats. Following testing, the brain of rat was analyzed using immunocytochemistry and immunofluorescence. The results revealed that iPSC-NPCs differentiated into neurons replacing the loss of hippocampus neurons, and iPSC-NPCs transplantation showed higher expression of glial fibrillary acidic protein (GFAP) and increased number of NeuN compared with the control group. Moreover, western blot analysis suggested enhanced expression of brain-derived neurotrophic factor (BDNF) in hippocampus tissue of iPSC-NPCs transplanted rats in comparison to the PBS group. Collectively, these findings showed that iPSC-NPCs could promote regeneration and motor function recovery in PTSD model.

1. Introduction

Post-traumatic stress disorder (PTSD) is a kind of stress-related emotional disorder which is usually caused by exposing to serious traumatic events such as severe injury, threat of death and sexual violation. The incidence of PTSD is relatively high when exposure to traumatic events. It was reported that over 70 % of the individuals worldwide had suffered at least one traumatic event. Among the traumatic events, injuries and accidents with 35 % lifetime prevalence were the most typical, and 17 % of the individuals were diagnosed with PTSD [1]. The psychopathological responses to PTSD are featured by memory impairments, anxiety, substance abuse disorder and fear. PTSD tends to result in mental disorders as well as immunological impairments including depression, stroke, myocardial infarction, even oncological diseases [2,3]. More importantly, these symptoms are associated with functional and structural changes in multiple regions of the brain [4]. At cellular level, PTSD is also related to a decrease of glial fibrillary acidic protein (GFAP) immunoreactive cells in the brain [5]. It is also shown that PTSD exhibits impaired brain-derived neurotrophic factor (BDNF) function and hippocampus neurons injury [6]. Generally, the available therapeutic option for PTSD is mainly pharmacological therapy, serotonin uptake inhibitors (SSRIs) and antidepressants are usually prescribed [7]. Long-term treatment with medicines is accompanied with serious adverse effects, and the risk-free rate must be evaluated thoroughly when prescribing them [8]. Traditional therapy of PTSD has not been fully successful due to side-effects of drug treatment, time and energy needed for psychotherapy. Drug treatment has also appeared insufficient benefit in the therapy of PTSD [9]. Hence, it is imperative to seek new approaches for PTSD.

Recent progress in induced pluripotent stem cells (iPSCs), which are generated by reprogramming somatic cells *via* inserting OCT4, SOX2, c- Myc and Klf4 genes [10], has provided new insights into brain development and the consequences of its dysfunctions in neurodevelopmental diseases. iPSC can be able to differentiate into any cell types including bone marrow stromal cells (BMSCs), neural progenitor cells (NPC) and embryonic cells (ESCs) in the body [11]. Among the various types of candidate cells, induced pluripotent stem cell-derived neural progenitor cells (iPSC-NPCs) hold great therapeutic potential for neurodevelopmental disorders, as they can replace the loss of neurons that have degenerated within the targeted region [12]. The stem cells can differentiate into neural lineages to replace the loss of hippocampal neuron and reconstruct the impaired hippocampal structure. Accumulating evidences have suggested that iPSC-NPCs exhibited promise for elevated tissue repair and functional recovery after neurogenic injury such as ischemic stroke [13], Huntington disease [14] and Parkinson disease [15].

Here, we explored the effects of iPSC-NPCs transplantation in PTSD rat model, aimed at

Abbreviations: PTSD, Post-traumaticstress disorder; GFAP, glialfibrillary acidic protein; BDNF, brain-derivedneurotrophic factor; iPSCs, inducedpluripotent stem cells; NPCs, neuralprogenitor cells.

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regeneration and function recovery. This study tested the efficacy of iPSC-NPCs transplantation as a therapy for PTSD by determining the differentiation capabilities, evaluating the effects on motor function and protein expressions 21 days after transplantation. This study was the first to investigate the regenerative properties of iPSC-NPCs in PTSD rat model.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (180− 220 g,) were purchased from the Animal Center of Jinling Hospital, Nanjing, China, and were kept in a standard 12/12 h light/dark cycle, with water and food pellets available *ad libitum*. All experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Committee of Animal Use and Protection of Jinling. The rats were sacrificed by decapitation. This research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review.

2.2. iPSCs culture and differentiation

Cell culturing of iPSCs and induction were performed as described [16]. Briefly, human iPSCs (Chinese Academy of Sciences, Shanghai) were cultured in embryonic stem cell (ESC) medium on feeder layers at 37 °C containing 5% CO_2 until colonies with typical human ESC morphology appeared. Individual colonies were dissociated mechanically and plated onto fresh medium for up to 8 passages before being adapted to feeder free conditions with TeSR-E8 medium.

When colonies reached 80 % confluency, iPSCs were dissociated and plated at a density of 5 × 10^5 cells in embryoid bodies (EBs) medium consisting of a mixture of 1% GlutaMAX, 1% N2, 2% B27, 1.25 μM dorsomorphin and 1% penicillin-streptomycin. Then, EBs were digested into single cells using Accutase® and plated at 2 × 10^6 cells per T25 with
NPCs medium supplemented with DMEM/F12, 1% B27, 1% penicillin–streptomycin, 10 ng/mL fibroblast growth factor and 10 ng/mL epidermal growth factor.

2.3. Establishment of PTSD rat model and transplantation of iPSC-NPCs

The animal experimental protocols were approved by the Ethics Committee of National Clinical Research Center of Kidney Disease, Jinling Hospital. Rats were randomly assigned into 6 groups (n = 8): control, PTSD, PTSD + PBS, PTSD + iPSC-NPCs at day 7, PTSD + iPSC- NPCs at day 14 and PTSD + iPSC-NPCs at day 21. The PTSD model was established according to previous study [17]. 14 days after injury, 1 × 10^6 iPSC-NPCs were transplanted into the hippocampus site by intracranial positioning. At different time points after transplantation, rats were sacrificed and their brains were dissected for immunocytochemistry analysis.

2.4. Immunocytochemistry of differentiated cells

Cells were fixed in 4% paraformaldehyde, blocked and permeabilized using 0.1 % Triton-X, 0.1 % Tween-20 and 2.5 % BSA in PBS. Then, cells were treated with primary antibodies against Tuj-1 (1:1,000, ab78078, Abcam) and GFAP (1:1,000, ab7260, Abcam) and counter- stained with AlexaFluor secondary antibodies. DAPI was added to stain Nuclei. Finally, cells were observed under a fluorescent microscope.

2.5. Cognitive functions analysis

2.5.1. Open field (OF) test

The open field activity of rats was quantified as described [18]. Briefly, rats were placed in the center of an open field (60 × 60 × 40 cm), let free-moving for 10 min, and all locomotor activities including total distance traveled, interest residence time and behavior modification were recorded automatically by monitoring system (XR-XZ301, Shanghai XinRuan Information Technology Co., Ltd, China). Prior to placing the next rat, the device was cleaned with 75 % ethanol.

2.5.2. Fear condition test

Rats were put in an experimental chamber (32 × 32 × 45 cm) with white walls, let free-moving for 3 min to familiar situations. Then, the rat was subjected to one tone-foot-shock pairing (tone, 30 s, 80 dB, 3 kHz; foot-shock, 2 s, 0.75 mA) and remained for 30 s after shock. The context-dependent test was examined at 24 h after training, which allowed the rat to explore in the same chamber for 5 min. The rat was placed in a new chamber with different color and shape for 3 min after 2 h, the training tone was delivered to evaluate tone fear conditioning (non-tone, 180 s; tone, 180 s, 80 dB, 3 kHz). The freezing time was tracking automatically by image monitoring system (XR-XC404, Shanghai XinRuan Information Technology Co., Ltd, China).

2.6. Immunofluorescence assay

The brain tissues were fixed with 4% paraformaldehyde, dehydrated by concentration of gradient (10 %, 20 %, 30 % and 5%), and then sections at 12 μm-thick was prepared for experiments. The neurons were treated with 4% paraformaldehyde after 3-time washing. All sections were blocked and incubated with primary antibodies against GFAP (1:1,000, ab7260, Abcam) and NeuN (1:1,000, ab177487, Abcam) at 4 °C overnight, followed by incubating with the secondary antibody (Alexa-Fluor 488, ab150077, Abcam) for 2 h. the nuclear was staining by DAPI (1:500, Bioworld, USA). The images were captured under a fluorescence microscope (Olympus, Japan).

2.7. Western blot

To analyze BDNF expression in the brain tissue after iPSC-NPCs transplantation, the brain tissues were isolated. Total protein was extracted using lysis buffer and the protein concentration was detected by BCA kit (Sigma-Aldrich, USA). Then, the protein

samples were separated on 10 % SDS-PAGE followed by transferred to PVDF membranes. The membranes were blocked with a mixture of 5% skim milk and 0.1 % Tween 20 in Tris-buffered saline followed by incubation with the primary antibodies against BDNF (ab203573, Abcam) and GAPDH (ab8245, Abcam) at 4 °C for 24 h. Then, the membranes were washed for 3 times and incubated with HRP-conjugated secondary antibody for 1 h at 37 °C. Finally, the protein bands were submitted to an ECL kit (Thermo Scientific) for visualization.

2.8. Statistical analysis

All the experiments were analyzed using GraphPad Prism 5.0. Data was expressed as the mean ± SD. Student's *t*-test was used to measure the differences between two groups, ANOVA analysis was used to compare the difference among multiple groups. $P < 0.05$ was indicated as statistically significant difference.

3. Results

3.1. Characterization of iPSC-NPCs in vitro

To ensure that iPSCs differentiated into NPCs, we differentiated the cells for 24 days. during neuralization, cell morphology changed from expanding colonies to neural rosettes after 14 days in induction. To detect whether iPSCs differentiated into neuronal cells, we labeled cells with Tuj-1 and GFAP, as markers of mature neuronal, to clarify whether iPSC-NPCs were able to differentiate after 14 days in differentiation medium. The results of immunocytochemistry analysis showed that NPCs expressed Tuj-1 and GFAP (Fig. 1), indicating induction to neural lineage.

3.2. iPSC-NPCs transplantation ameliorated the cognitive deficits of PTSD rats

To explore the role of iPSC-NPCs transplantation in cognitive deficits including locomotor activity and anxiety, an open field test was performed. Open field test, also known as open box test, is a method to evaluate the autonomous behavior and tension of experimental animals in new and different environments. It contions total distance, interest area stay time and behavior modification. Total distance – reflects the movement of rats. For example, depressed and feared rats would have much less exercise distance and would be more likely to stay in a corner of an open field. Interest area stay time – reflects the anxiety and fear of the rats. Behavior modification mainly reflects the inquiry behavior of rats. As shown in (Fig. 2), PTSD rats exhibited significant decrease in distance, interest residence time and behavior modification compared with the control group, whereas there was no remarkable difference between iPSC-NPCs transplantation after 7 days and PTSD groups. However, PTSD rats treated with iPSC-NPCs transplantation after 14

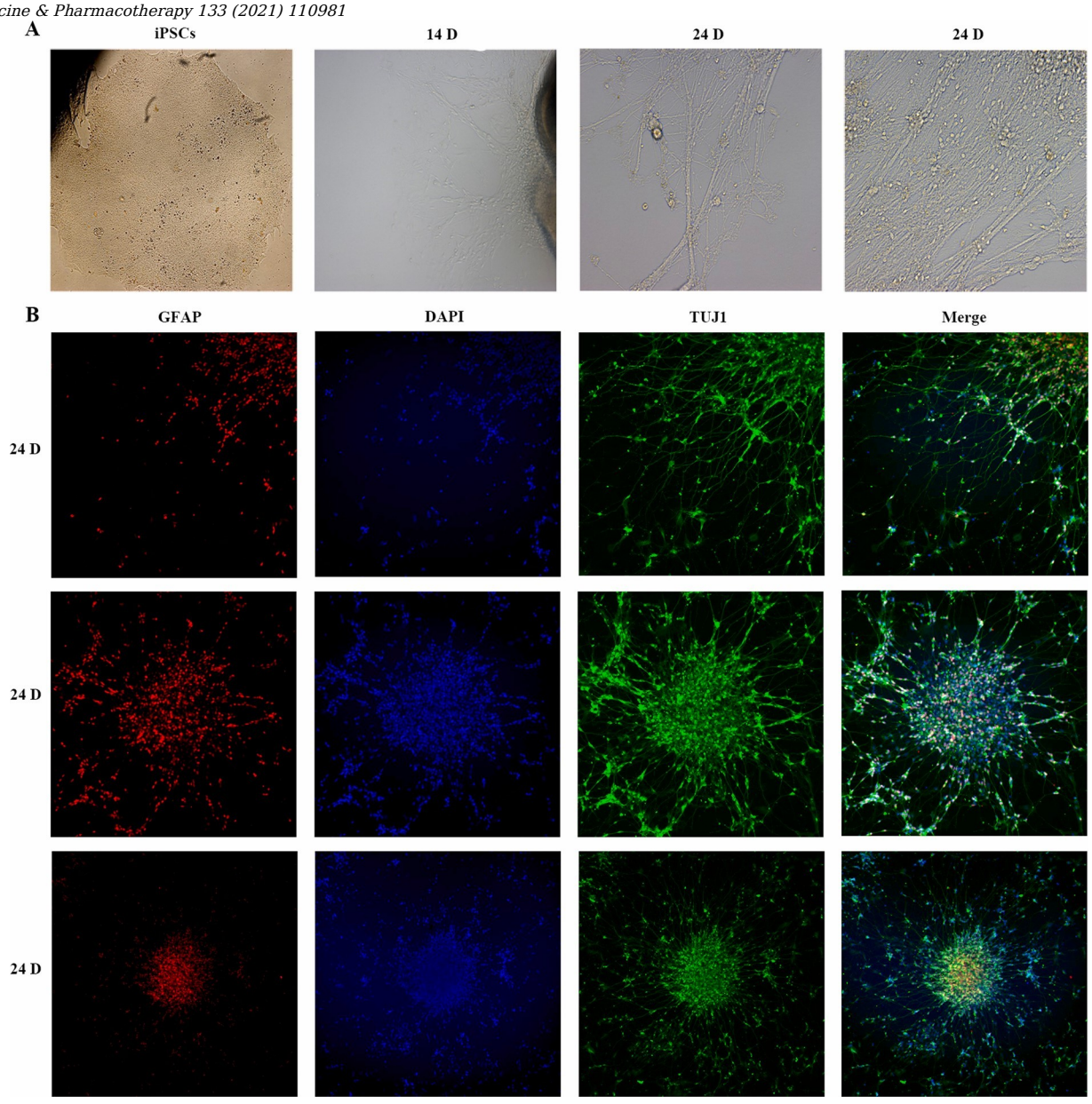

Fig. 1. Characterization of iPSCs. (A) Cell morphology of iPSCs after induction. (B) Immunofluorescent staining images of Tuj-1 (green), GFAP (red) and DAPI (blue) of iPSC-NPCs. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

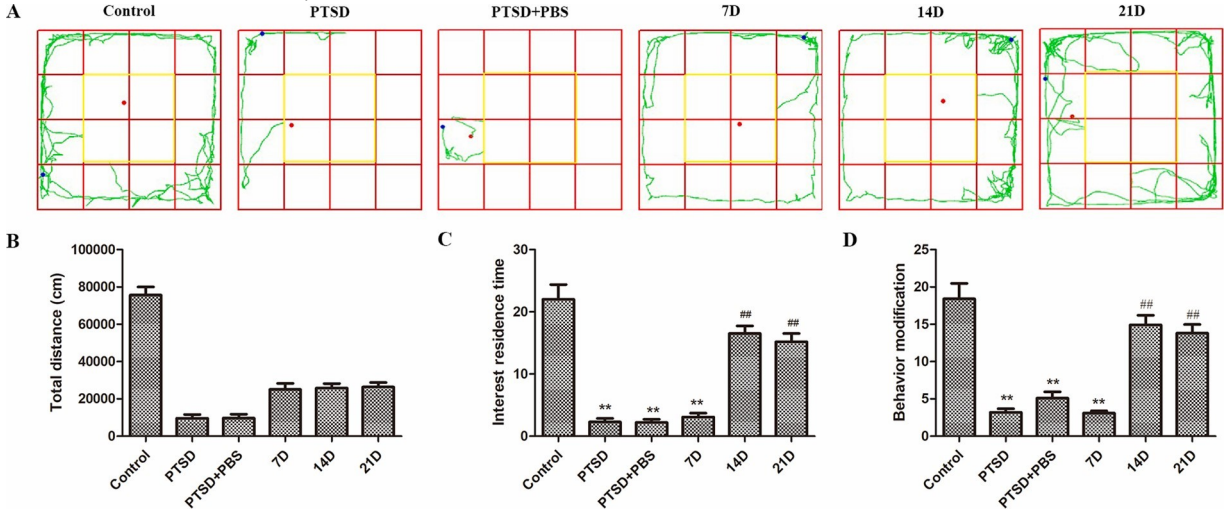

Fig. 2. The effects of iPSC-NPCs transplantation on locomotion, anxiety-like symptoms in PTSD rats. The green lines are the routes of the rats, The red line is the range of the rats. (A) The movement track of rats under different conditions. (B) Total distance traveled (C) The interest residence time (D) The behavior modification of rats under different treatment. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

days and 21 days exhibited distinct increase in interest residence time and behavior modification. These results indicated that short-term iPSC- NPCs transplantation did not lead to detectable changes on locomotor activity and anxiety, while long-term treatment improved the cognitive dysfunction, and the iPSC-NPCs transplantation can improve the cognitive dysfunction of PTSD.

To further evaluate the effects of iPSC-NPCs transplantation on cognitive function, fear conditioning test was conducted. Freezing is a kind of animal defense behavior. It means that animals do not have any other activities except breathing. It is considered a reliable indicator of rodent fear. By contrast, PTSD rats exhibited increased freezing time in the context test, while iPSC-NPCs transplantation significantly reversed the influence with decreased freezing time (Fig. 3). These data revealed that iPSC-NPCs transplantation could ameliorate the hippocampus- dependent memory in PTSD rats.

3.3. iPSC-NPCs transplantation increased astrogliosis

Immunostaining analysis was performed to explore whether iPSC- NPCs transplantation affected the expression of GFAP. As depicted in Fig. 4, there was a significant increasing in the number of GFAP expressing astrocytes in the iPSC-NPCs group, at day 21 post- transplantation compared with the PBS group. This increasing was also observed in the iPSC-NPCs group at 7 days, when compared to the control group. Additionally, iPSC-NPCs group at 21 days showed a remarkable increasing in expression of GFAP in comparison to the control group. Collectively, treatment with iPSC-NPCs following PTSD confirmed an increase in astrogliosis, especially 21 days post- transplantation.

3.4. iPSC-NPCs enhanced expression of mature neurons

To further determine the effects of transplanting iPSC-NPCs on neuronal maturation in the hippocampus, NeuN as a marker of mature neurons was detected. When compared to the control group, a significant decrease in NeuN expression cells was observed in PTSD rats. However, there was a distinct increase in NeuN levels in iPSC-NPCs transplantation group, in comparison to post-PBS group (Fig. 5). Overall, transplantation of iPSC-NPCs post-PTSD suggested that it could increase neurogenesis of hippocampus.

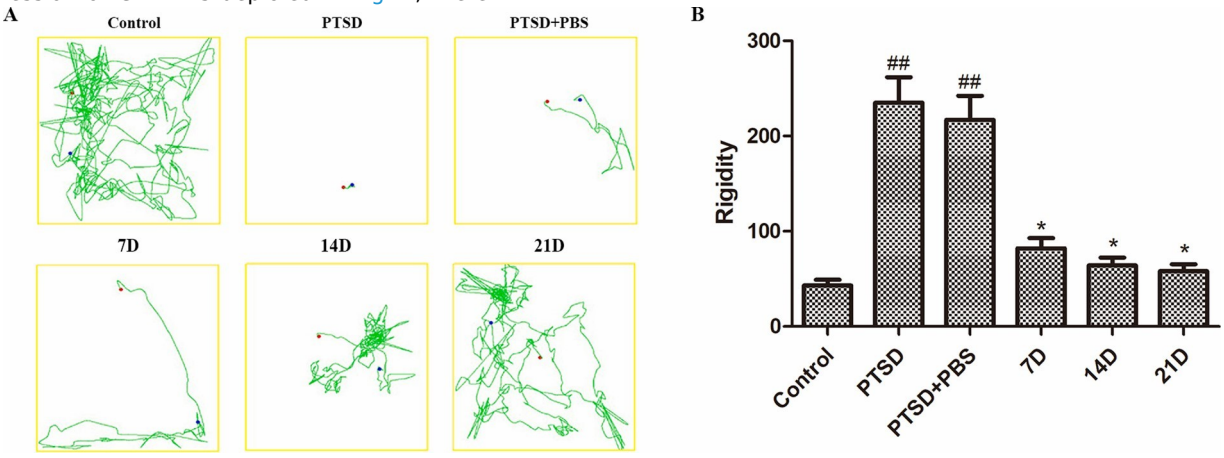

Fig. 3. (A) The movement trail of rats under different conditions was detected by fear condition. The green lines are the routes of the rats, The red line is the range of the rats. (B) The statistical data of rigidity was shown. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

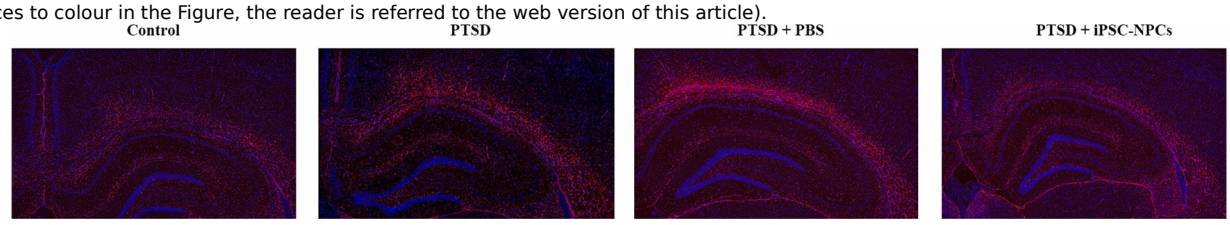

Fig. 4. The expression of GFAP in the hippocampus of PTSD rats was detected by immunofluorescence assay.

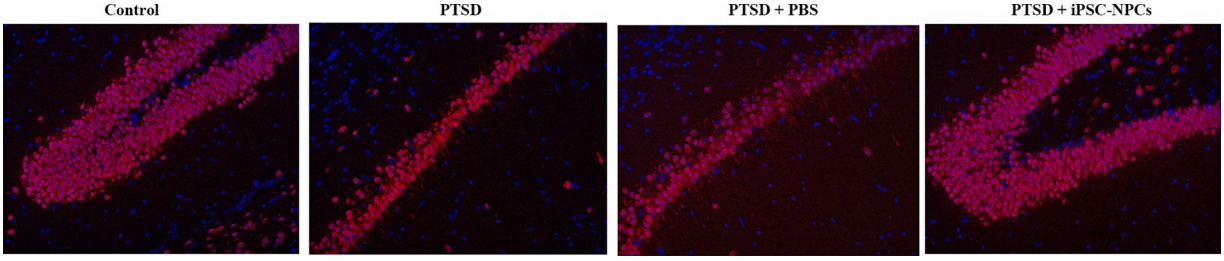

Fig. 5. The effects of transplanted NPCs on mature neurons of hippocampus were detected by immunofluorescence assay.

3.5. iPSC-NPCs transplantation increased BDNF level in the brains of PTSD rat

To investigate whether iPSC-NPCs transplantation affected the expression of BDNF in the brain, western blot analysis was employed. As shown in Fig. 6, rats following PTSD exhibited reduction in the expression of BDNF, whereas the reduction was reversed by transplanting iPSC-NPCs at day 14 and 21 as evidenced by increasing expressions of BDNF. A slight increasing of BDNF expression was also observed in the iPSC-NPCs group at 7 days, when compared to the PTSD group. These results demonstrated that iPSC-NPCs transplantation could promote functional recovery after PTSD at least partially through upregulating BDNF.

4. Discussion

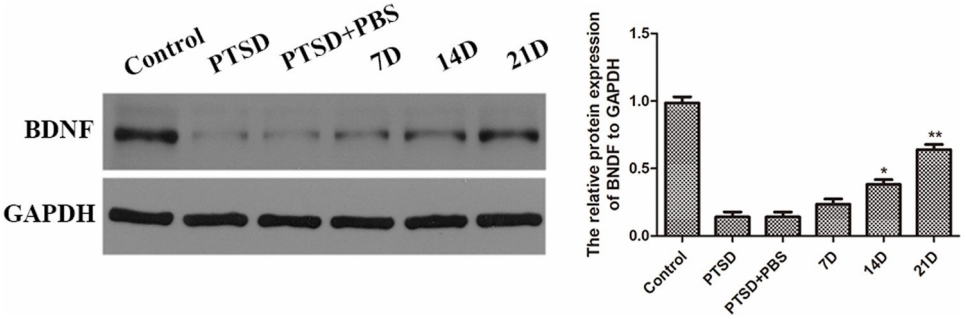

Fig. 6. The protein level of BDNF in the brain was detected by Western blot.
The expression of BDNF in PTSD was reduced and in IPSC-NPCs after 14 days and 21 days was increased.

Cell therapy remains one of the most promising strategies for the therapy of neurological disorders. However, many of the existing available cell systems have been demonstrated to present with heterogeneity, serious hurdles and ethical concerns [19]. Stem cells characterized by inherent expansion ability and functional plasticity could alleviate these disadvantages by replacing dead cells and exerting anti-inflammatory [20]. Stem cell transplantation using mesenchymal stem cells (MSCs), iPSCs and neural stem/precursor cells (NS/PCs) has been proposed as a prospective therapy for brain injury [21]. iPSCs have become the focus in the field of stem cell research and have been widely used in various fields such as autologous cell transplantation, tissue engineering and drug development [22,23]. Due to self-renewal and pluripotent potential, iPSCs have the capability to differentiate into multiple types of mature cells including NPCs *in vitro* [24]. NPCs hold a great potential for the treatment of neurological disorders because of their capacity to integrate into the host tissue, thereby contributing to the replacement of damaged cells [25]. NPCs transplantation had been shown to exhibit the neurotrophic effects and improved behavioral outcomes on cerebrovascular disease [26]. NPCs transplantation was also demonstrated to have neuroprotective effects in rodent models of spinal cord injury [27]. Moreover, NPCs over-expressing BDNF and GDNF could provide better neuroprotective effects on numerous neuronal injury diseases such as stroke and Parkinson [28].

PTSD, marked psychosocial and occupational impairment, is a considerable burden to healthcare because of its severity and chronicity [29]. Solid evidence has suggested that the development of PTSD is associated with cognitive deficits and memory dysfunction [30]. The present study showed that iPSCs were efficiently differentiated into NPCs *in vitro*. iPSC-NPCs transplantation could improve cognitive dysfunction in PTSD rats. Moreover, iPSC-NPCs transplantation could lead to enhanced neurogenesis of hippocampus with increased expression of BDNF. To our knowledge, this study is the first one to employ iPSC-NPCs transplantation as a readout of neuronal function following PTSD.

GFAP, as an astrocyte protein, is reduced following brain injury, which might be due to the fact that astrocytes provided trophic support to maintain the recovery processes [31]. To verify whether iPSC could differentiate into neurons to replace lost cells, we demonstrated that iPSC could be differentiated into neural progenitors. In addition, iPSC-NPCs could differentiate into neurons expressing several neuronal markers including Tuj-1 and GFAP. Moreover, transplanted iPSC-NPCs were able to differentiate into mature neurons and glial cells *in vivo* indicating that these cells could provide rebuilding mediums for tissue repair in PTSD. Our findings were in line with published report verifying that iPSC-NPCs transplantation is an available treatment option for brain injury [32]. Prior study revealed that the number of glia in the amygdala and GFAP-immunoreactivity in the hippocampus were decreased in depressed patients. Besides, previous research also demonstrated that stress induced great increase in the expression of GFAP in the hippocampus [33]. However, decreased expression of GFAP in PTSD rats in our study indicated the loss of hippocampus glial cells.

The present study revealed that PTSD rats produced representative cognitive dysfunction such as weakened locomotor activity, anxiety-like behavior and memory alterations, which was in line with previous study that PTSD had negative influence on motor and cognitive functions [34]. Nevertheless, when iPSC-NPCs were transplanted into PTSD rats for 21 days, this cognitive impairment was attenuated, as indicated by increased total distance, interest residence time and decreased freezing time. However, short-term transplantation of iPSC-NPCs into PTSD rats did not change their

behavior significantly. These results suggested that long-term iPSC-NPCs transplantation could ameliorate cognitive impairment induced by an intense stress experience and provide behavioral evidence to support the use of iPSC-NPCs transplantation for the treatment of PTSD.

The hippocampus is considered as an important region in PTSD because it plays a critical role in the neuroendocrine stress response as well as memory alterations [35]. It has been shown that atrophy of hippocampus is one of the common pathologic changes in PTSD patients [36]. PTSD could lead to hippocampus morphological changes including decreased neurogenesis and neurons loss [37]. Subsequently, we evaluated the effects of iPSC-NPCs transplantation on neurogenesis by using NeuN as a marker of mature neurons. Although it took 14 days for the new progenitors to become functionally mature neurons, the PTSD groups showed a decrease in NeuN expression, whereas the group transplanted with iPSC-NPCs confirmed a significant increase in NeuN expression. Our results were in line with the previous report that exogenous NPCs were able to rescue host neurons [38].

Treatment benefits of cell transplantation are mainly focused on cellular replacement such as neuronal differentiation and integration, or release of trophic factors such as BDNF [39]. The role of BDNF as neurotrophin as well as neuroprotector make it play an important role to modulate the acute response to neurotrauma. It has been reported that the expression of BDNF in the hippocampus was significantly decreased in animals following PTSD [40]. To identify iPSC-NPCs-related mechanisms of memory improvement, the effects of iPSC-NPCs on the protein expression of BDNF in the hippocampus, which was crucial for hippocampus-dependent learning and memory, were investigated. PTSD-induced memory deficits caused obvious reduction in BDNF expression in the hippocampus and led to poor performance in hippocampus-dependent tasks [41]. There was also evidence revealing that BDNF function and production were impaired in patients after PTSD [42]. In our study, it was found that long-term iPSC-NPCs transplantation significantly inhibited decrease in BDNF expression induced by PTSD, which was consistent with previous publication that transplanted NPCs could secrete multiple neurotrophic factors [43]. The results manifested that the beneficial effects of iPSC-NPCs transplantation were mediated by an increase in BDNF expression.

5. Conclusions

In summary, this study suggested that iPSC-NPCs had preventive effects against traumatic stress-induced cognitive dysfunction in a PTSD rat model. Moreover, the study also revealed that iPSC-NPCs transplantation was associated with the changes in neurogenesis and expression of BDNF in the hippocampus. Overall, the present study provides evidences to support the effectiveness of iPSC-NPCs transplantation for PTSD treatment.

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Declaration of Competing Interest

The authors report no declarations of interest.

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